third step at 70°C for 40 sec. PCR ended with 8 min at 70°C. Hybridization was performed using the TwinCubator at a temperature of 45°C. The denaturation solution was mixed with 20 µl of the amplified sample and submitted to the usual protocol for hybridization.

In order to assess positive and negative bands, each DNA strip was stuck on an evaluation sheet after the hybridization, and a template was aligned side by side of the respective strip, with at the top the conjugate control band and at the bottom the coloured M marker band. Positive control bands, i.e. that should appear positive to make the test valid, were the conjugate control, the amplification control, the identification control for the *M. leprae* species and amplification controls of the *rpoB*, *folP1* and *gyrA* genes.

Interpretation was as follows for each gene/antibiotic: the strain was predicted to be susceptible when all WT bands were positive and all MUT bands were negative; the strain was predicted to be resistant when at least one MUT band was positive or at least one WT band was negative.

DNA extraction and reference PCR-sequencing

PCR sequencing was performed routinely and prospectively in the frame of NRC-Myc activities, as individual susceptibility to rifampin (*rpoB*) and dapsone (*folP1*) for all the 112 biopsies whereas ofloxacin susceptibility was tested for 52 biopsies. PCR sequencing was performed specifically in the frame of the present study for the 8 reference strains.

Total DNA was extracted from biopsies containing *M. leprae* following the heat-shock procedure [24]. DNA was subjected to three PCRs, one amplifying the RRDR in *rpoB* gene, one the DRDR in *folP1* and one the QRDR in *gyrA*, as previously described [10,25]. Typical reaction mixtures (50 µl) contained 1× reaction buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1 µM of each primer (Proligo France SAS), 1.25 U of *Taq* polymerase (Q-Biogene, Illkirch, France) and 5 µl of DNA extract. PCR-amplified fragments were purified by using Montage™ PCR Centrifugal Filter Devices (Millipore, Molsheim, France) and sequenced by the dideoxy-chain termination method with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France). The oligonucleotide primers used for DNA sequencing were those used for PCR. The nucleotide and deduced amino acid sequences were analyzed with the Seqscape v2.0 software (Applied Biosystems).

Antibiotic susceptibility testing in the mouse

Animal experiments were performed in accordance with prevailing regulations regarding the care and use of laboratory animals by the European Commission. The experimental protocol was approved by the Departmental Direction of Veterinary Services in Paris, France.

The *M. leprae* strains were subjected to the mouse footpad susceptibility testing that included 10 untreated Swiss mice as a control group, a rifampin-treated group of 8 mice and a dapsone-treated group of 30 mice as described previously [17],[20]. Dapsone susceptibility testing was stopped in 2001 because of new governmental regulation for antibiotic-free animal feeding. An additional group of 8 ofloxacin-treated mice was inoculated, as described in [7], for the biopsies sampled in patients who have been treated by fluoroquinolones.

Evaluation of the diagnosis performances

The results of the GenoType LepraeDR test were compared to those of the PCR sequencing method for all the 120 *M. leprae* strains (60 in the case of ofloxacin and *gyrA*).

The results of the GenoType LepraeDR test were also compared to the results of the mouse footpad model for *M. leprae* strains that yielded interpretable susceptibility results, i.e. 84 strains tested in vivo for rifampin susceptiblity, and among them 56 for dapsone susceptibility and 5 for ofloxacin susceptibility.

Results

Performances of GenoType LepraeDR for detection of *M. leprae*

The DNA strip tests were validated with regard to the *M. leprae* identification band, which was positive with an intensity equal or higher than that obtained with the universal positive control, demonstrating the presence of *M. leprae* DNA. Thus, the overall sensitivity of GenoType LepraeDR for detecting *M. leprae* was 100%.

Analytical specificity tested with either DNA from another mycobacterial species (n = 19) or negative skin biopsies (n = 10) was 100% since no positive signal was obtained for the *M. leprae* identification band. However, hybridization was observed for DNA from *M. intermedium* and *M. malmoense* with two of the wild type *rpoB* bands, due to a high identity between the *rpoB* genes of these mycobacterial species.

Performances of GenoType LepraeDR for detecting mutations in the genes involved in antileprosy drug resistance

The mutations found in the *M. leprae* strains by PCR-sequencing are listed in the Table 2. Representative results of the DNA strip tests are shown in Figure 1 for resistant strains and in the Figure 2 for susceptible strains.

The results of the DNA strip test were concordant with those of PCR sequencing for all the 16 *rpoB* mutations conferring rifampin resistance (Table 3). We observed a positive signal at probes rpoBMUT2 for the 10 strains harboring the mutation S456L and at rpoBMUT1, for the strain harboring the H451Y mutation, since these mutations are present onto the strip as a mutated probe. As expected for these strains, no signals were observed for the wild type probes rpoBWT4 and rpoBWT3, respectively. For the others mutations, the test detected the *rpoB* mutation through the lack of hybridization with the wild type probes that include the mutated codon (Table 1), e.g. with rpoBWT4 for the two strains harboring the mutation S456M or S456F, with rpoBWT2 for the strain with the mutation Q438V, rpoBWT1 and rpoBWT3 for the strain harboring the two mutations G432S + H451D and

Table 2. List of mutations present in the *M. leprae* resistant strains.

Mutations in the region determining resistance in the following genes (N strains)		
rpoB	*folP1*	*gyrA*
S456L (10)	P55L (8)	A91V (4)
S456F (1)	P55R (3)	
S456M + L458V (1)	T53I (7)	
H451Y (1)	T53A (3)	
G432S + H451D (1)	T53V (1)	
T433I + D441Y (1)		
Q438V (1)		

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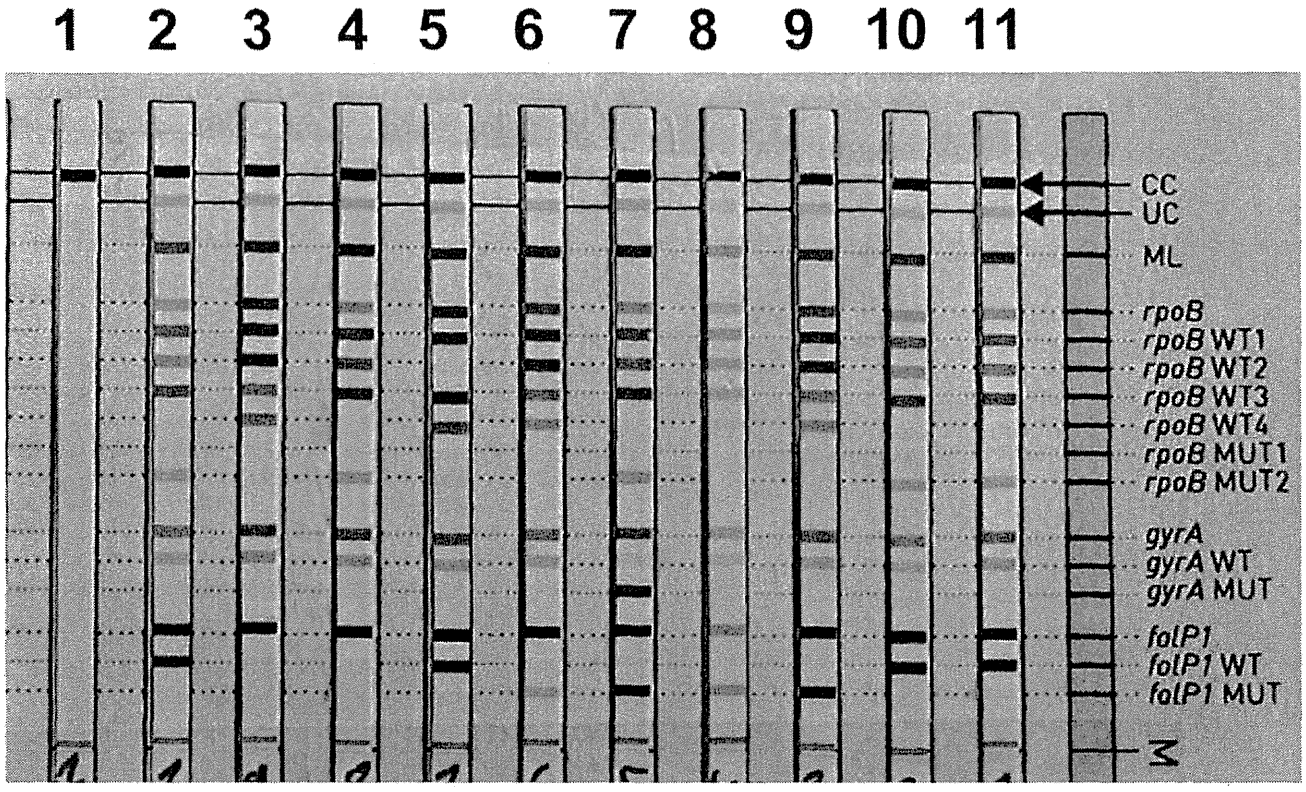

Figure 1. Mutations conferring resistance in *Mycobacterium leprae* are detected by the GenoType LepraeDR DNA strip test. Lane 1 is a negative control (only the CC band). Lanes 2 to 11 showed various profiles for resistant strains: lane 2, *rpoB* mutation S456L with wild type *gyrA* and *folP1* alleles; lane 3, wild type *rpoB* and *gyrA* alleles with a *folP1* mutation to be defined; lane 4, *rpoB* mutation S456L with a wild type *gyrA* allele but a mutation in *folP1*; lane 5, *rpoB* mutation (Q438V) with wild type *gyrA* and *folP1* alleles; lane 6, wild type *rpoB* and *gyrA* alleles with a P55L mutation in *folP1*; lane 7, *rpoB* mutation S456L with a A91V *gyrA* mutation and a P55L mutation in *folP*; lane 8 and lane 9, wild type *rpoB* and *gyrA* alleles with a P55L mutation in *folP1* ; lane 10 and lane 11, *rpoB* mutation S456L with wild type *gyrA* and *folP1* alleles. The numbering system used is that of the *Mycobacterium leprae* genome strain NT (n°NC 002677).
doi:10.1371/journal.pntd.0001739.g001

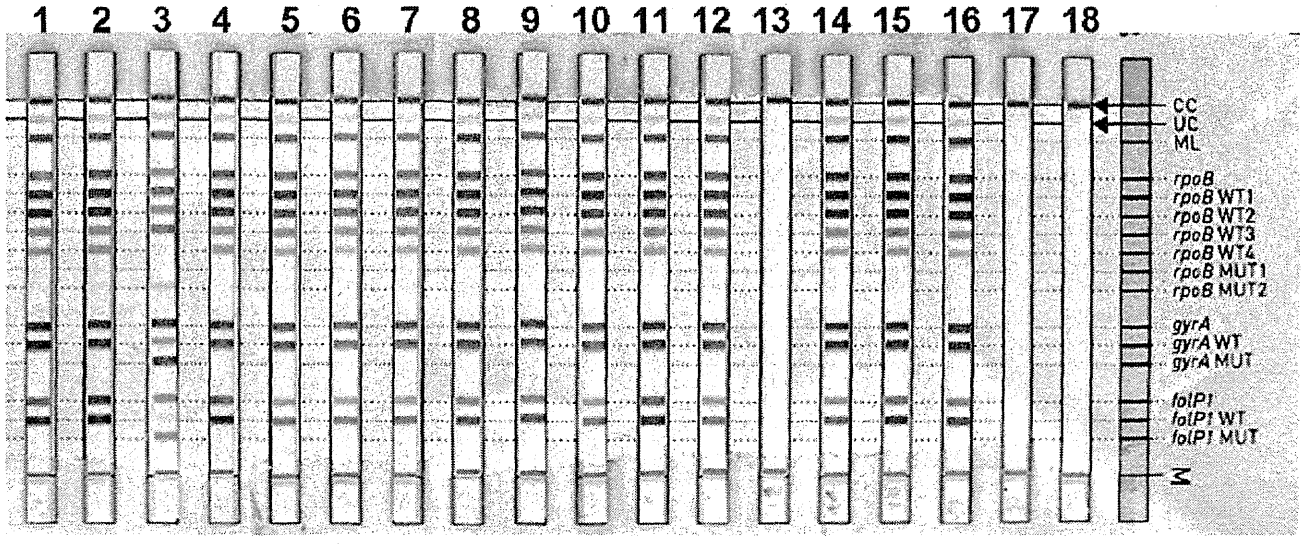

Figure 2. *Mycobacterium leprae* susceptible strains showed a wild type profile in the GenoType LepraeDR test. Lane 1 to 16 (except lane 8) showed wild type profiles for susceptible *M. leprae* strains. Lane 8 showed a multiresistant profile with mutations in *rpoB*, *gyrA* and *folP1* genes. Lanes 17 and 18 showed result of negative controls.
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Table 3. Concordance of results for the DNA strip test (GenoType LepraeDR) and the susceptibility phenotypic and genotypic pattern of antibiotic resistance for the *M. leprae* strains studied.

M. leprae strains	N diagnosis tests with interpretable results			Concordance GenoType LepraeDR N strains (%)	
	In vivo susceptibility testing*	PCR sequencing	DNA strip test	versus in vivo Susceptibility testing	versus PCR sequencing
Total tested for at least one antibiotic	84	120	120	84 (100%)	120 (98%)
Rifampin resistant	13	16	16	13 (100%)	16 (100%)
Rifampin susceptible	71	104	104	71 (100%)	102** (97%)
Dapsone resistant	8	22	22	8 (100%)	22 (100%)
Dapsone susceptible	48	98	98	48 (100%)	98 (100%)
Ofloxacin resistant	1	4	4	1	4
Ofloxacin susceptible	4	56	56	4	56 (100%)

*For strains growing in vivo and yielding interpretable susceptibility results. Tests were stopped for dapsone due to new regulation for antibiotic animal feeding. Tests for ofloxacin were restricted to patient with previous treatment by fluoroquinolones.
**including two strains with a mutation at codon 447: Ser447Cys for one strain and a silent mutation for the second strain (see text for details).
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rpoBWT1 and rpoBWT2 for the strain harboring the two mutations T433I + D441Y. For two strains carrying a mutation at the codon 447, they were not detected by the DNA strip test since no probe spanning this codon is included in the strip because this mutation was not known to confer resistance. The first of these strains showed a silent mutation and the second showed a mutation leading to the substitution S447C. Although the latter strain appeared susceptible to rifampin in the routine mouse footpad testing, we repeated this test using decreasing dosages of rifampin in order to be sure that the S447C mutation does not confer resistance in *M. leprae* as a similar mutation does in *M. tuberculosis* [26], even at a low level. For this purpose, three groups of mice (10 mice per group) were treated by 10 mg/kg (normal dosage), 5 mg/kg or 2.5 mg/kg rifampin. Growth was not observed in any of these groups but occurred in the control untreated group, demonstrating that the strain was really susceptible to rifampin and that the S447C mutation was not conferring resistance. Moreover, the patient, who was an immigrant from Senegal, was cured after being treated by the standard MDT, i.e. the combination of rifampin, dapsone and clofazimine. For the other 102 other strains, no mutations were detected by the RRDR sequencing in *rpoB* and the DNA strip test.

Concordance was observed between the DRDR sequence in *folP1* and the DNA strip test: 22 strains with a *folP1* mutation involved in dapsone resistance and 98 strains with a wild-type *folP1* sequence (Table 3). Hybridization was observed with the *folP*1 MUT probe for the 8 strains with the *folP1* P55L mutation. For the 14 strains harboring other mutations at codon 55 (P55R) or at codon 53 (T53I, T53A, T53V), there was no signal with the wild type probe, showing that there was a mutation.

Finally, we observed a concordance between the QRDR sequence in *gyrA* and the DNA strip test results: 56 strains with a wild type sequence showed a *gyrA* WT band and the four strains with the mutation A91V showed the *gyrA* MUT band (Table 3).

Concordance between susceptibility phenotype and genotype determined by the DNA strip test

Concordance was observed between the phenotypic susceptibility results assessed by the mouse footpad model and the genotype detected by the GenoType LepraeDR test. Results are detailed in Table 3 with regard to the antibiotic tested.

Concordance between rifampin phenotypic susceptibility in vivo and the results of GenoType LepraeDR was obtained for all the 84 strains tested. Thirteen rifampin-resistant strains showed either the rpoBMUT1 band (S456L) for 9 strains, or the absence of at least one rpoB WT band for the remaining 4 strains, which indicated a mutation in the RRDR. The exact nature of the *rpoB* mutation was further identified by PCR-sequencing. All the 71 susceptible strains were founded susceptible by the DNA strip test since all the rpoB WT bands were positive and all of the MUT bands were negative.

Concordance between dapsone phenotypic susceptibility and detection of *folP1* mutation by the DNA strip test was obtained for the 48 susceptible and the 8 resistant strains. For all the resistant strains, the folP WT band was negative, indicating a mutation in the DRDR. The folP MUT band was positive for two of these strains, indicating a mutation P55L. In the 6 remaining strains, the exact nature of the *folP* mutation was identified by PCR-sequencing. For the 48 dapsone-susceptible strains, the folP1 WT band was positive and the MUT band was negative

Finally, results of ofloxacin phenotypic susceptibility were concordant with the results of *gyrA* obtained by the DNA strip test for the five strains tested in the mouse footpad: one was resistant and showed a positive gyrA MUT band (mutation A91V) with a negative WT band, and the four susceptible strains showed a positive gyrA WT band and a negative MUT band.

Discussion

Leprosy, after centuries of endemicity when the disease lasted the whole patient life due to a lack of efficient treatment, became a curable disease by combining rifampin and dapsone into a multidrug therapy regimen [2]. Consequently, a dramatic decrease in the prevalent active cases occurred during the two last decades. However, the incidence rate did not decrease showing that leprosy is still an actively transmitted disease [1]. Acquired resistance has been observed for each of the antileprosy drugs following their successive introduction as antileprosy agent [27,28]. Multidrug resistant strains resulting from the accumulation of distinct resistant traits have been described in several endemic regions [7,22]. Proportions up to 80% of secondary resistance (patients previously treated) and 40% of primary resistance (patients never treated) to dapsone and up to 40%

secondary resistance to rifampin, have been reported through local and limited studies [28,29,30]. Since *M. leprae* is not growing in vitro, it is not possible to measure resistance rates at large scale in endemic countries. Even in highly specialized leprosy centers where the animal model has been set up, it is nowadays very difficult to sustain animal facilities because of ethic rules and safety measures. Molecular detection of resistance to antileprosy drugs has been introduced since genetic bases of resistance were deciphered by expert scientific labs in France, US and Japan Cambau 1997 [10,11,12,31]. We previously showed that mutations in the target genes in clinical *M. leprae* strains were associated with acquired resistance demonstrated by in vivo drug susceptibility testing: in *rpoB* for rifampin resistance, in *folP1* for high and medium level dapsone resistance, and in *gyrA* for ofloxacin resistance [7,17,20]. These studies demonstrated concordance between genotypic and in vivo phenotypic results. Therefore, in-house molecular detection is being used for individual diagnosis of leprosy cases in countries where PCR sequencing is affordable [15,31,32,33,34,35,36].

Following years of using various in house molecular methods to rapidly detect for drug resistance in *M. tuberculosis*, particularly to detect for multi-drug resistant cases, i.e. cases resistant to isoniazid and rifampin that cannot be cured by the standard regimen, standardized and commercially available kits, such as the line probe assays, InnoLiPA Rif.Tb and GenoType MTBDR, and more recently GeneXpert RifTB, have been introduced and are recommended in low-income but highly epidemic countries (www.who.int/tb/strategy/en/).

WHO launched in 2008 a programme of surveillance of drug resistance in leprosy using molecular methods relying on a handful of national and supranational reference laboratories. First results obtained for cases reported in 2008, 2009 and 2010, showed that rifampin, dapsone and fluoroquinolone resistance were described but the resistance rates varied from 0 to 10% [37]. This needs confirmation at a larger scale and for an extended time. However this showed that the rates of resistance to antileprosy drugs can be measured by using molecular methods.

The DNA strip technology has been developed as GenoType kits and applied to the molecular detection of antibiotic resistance in various infections such as tuberculosis and *Helicobacter pylori* diseases [19,38]. This approach has been shown to be easy to use, requiring only a classic thermocycler and a hybridization chamber at a constant temperature of 45°C. This is the reason we choose to develop a standardized test based on the DNA strip technology able to detect for molecular detection of resistance in leprosy.

The new test, GenoType LepraeDR, was evaluated by systematically testing 120 *M. leprae* strains studied for genotypic and phenotypic characters of resistance [17,20,22]. The results yielded by the test were shown to be 100% concordant with those of the in vivo susceptibility testing whereas the results of PCR sequencing was 98.3% for rifampin, 100% for dapsone, and 100% for fluoroquinolones. Moreover, the two *rpoB* mutations not detected by the test, located at the codon 447, a codon not included in the test, were in fact not conferring rifampin resistance.

We focused deliberately the present evaluation on AFB-positive specimen from multibacillary leprosy cases for two reasons: (i) first the AFB positivity represents a major clue in leprosy diagnosis that allows concentrating subsequent tests on mot probable cases, an important point in low income countries and (ii) second, the risk of developing acquired resistance by selection of resistant mutants are highest in multibacillary cases. We did not evaluate the performances of the test on either AFB-negative specimen nor on specimen other than skin biopsies (e.g. nasal wabs). The specificity of the test with regard to other mycobacterial species involved in skin infections was assessed for Buruli ulcer and infections due to *M. marinum*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. terrae* and other less common mycobacteria. Because of the high identity of the *rpoB* gene between some mycobacterial species, the results of resistance mutation in *rpoB*, *gyrA and folP* genes by the test can be interpreted only when the test identifies the species as *M. leprae* (positive ML band).

Various other methods have been described to detect mutations in *rpoB*, *gyrA* and *folP* such as PCR sequencing, heteroduplexes, and DNA array [13,14,15,16,18]. There were mostly used in large laboratories affiliated to Universities of high income countries and collecting strains from endemic countries [34,39]. Since the reverse hybridization technology is already used in several countries endemic for tuberculosis, the same technology could be also used for the diagnosis of resistance in leprosy in countries where leprosy is still a preoccupying disease, with two objectives: (i) diagnosing resistance at the individual level and (ii) assessing rates of secondary and primary resistance in collaboration with health authorities [1,37]. Although leprosy is now diagnosed in the field using clinical findings only and no laboratory support is available, such a test can be used complementary to the clinical diagnosis of multibacillary leprosy for (i) relapse cases, especially those who have not been treated by MDT, i.e. before 1982, and (ii) survey of resistance in new cases in defined areas or periods for epidemiological surveillance on the behalf of leprosy public health programmes. Therefore the specimen can be send to a regional lab, especially one used to similar molecular test detecting resistance in tuberculosis. In addition, clinical microbiology laboratories in high income countries, which have usually moderate expertise in leprosy diagnosis and resistance detection, would appreciate the robustness of the test, and such a test can help in diagnosing cases from immigrants or national intertropical territories [40,41]. Using this technology routinely at the French National Reference Center for mycobacteria during the last two years, we diagnosed 35 cases of leprosy in patients living in France and detected 4 cases with dapsone resistant strains (*folP1* mutations as P55L in 3 strains and T53A in one strain) and 1 case with an ofloxacin resistant strain (*gyrA* A91V mutation) (data not shown). These results, obtained independently of the present evaluation, support the practical interest of this technology.

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Author Contributions

Conceived and designed the experiments: EC LTK VJ. Performed the experiments: ACN LTK MM. Analyzed the data: EC VJ. Contributed reagents/materials/analysis tools: LTK MM ACN. Wrote the paper: EC VJ.

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Genotyping of *Mycobacterium leprae* in Myanmar and supposed transmission mode

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The polymorphism of TTC repeats in *Mycobacterium leprae* was examined using bacilli from slit skin samples of leprosy patients attending at Central Special Skin Clinic, Yangon General Hospital and nasal swabs of their contacts to elucidate the possible mode of leprosy transmission. It was found that bacilli with different TTC genotypes were distributed among same household contacts and also harbored bacilli in patients were different TTC genotype from that harbored on the nasal mucus of the healthy contacts. Genotypes of TTC repeats were found to differ between husband under treatment and his wife and also mother under treatment and her sons living in same house. This study revealed that TTC genotype of bacilli harbored by household contacts was different with the TTC genotype by index cases. These results indicate that the family members get transmission from outside the dwellings rather than from commonly supposed their MB index cases. There might have been some infectious sources to which the populace had been commonly exposed outside the dwellings.

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection. It has long being believed that the source of infection is untreated multibacillary leprosy patients. It has also been predicted that multidrug therapy (MDT) with strong bactericidal antibiotics (such as rifampicin) would reduce the source of infection and consequently interrupt further transmission to others. However, the number of new cases has shown no substantial decline especially other than India. It is reported that about 200,000 to 30,000 new cases are continuously found in the world every year (1), which

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suggests that the transmission of leprosy bacilli still occurs, especially in countries of endemicity. Elucidation of the mode of transmission would be essential to reduce newly transmitted cases. The differentiation of strains of leprosy bacilli by genomic polymorphism might be of great value in efforts to understand the mode of transmission of the disease. The range of molecular techniques for epidemiological analysis has expanded in recent years, and there are now many genotypic methods that allow a high level of discrimination between bacterial strains (references 2, 3, 4). Shin *et al.* discovered a genomic divergence of *M. leprae* by the variation of TTC repeats (5) and subdivided 34 isolates into 15 subtypes. Genotyping according to the TTC repeats for fragments amplified by PCR seemed to be feasible for molecular epidemiological analysis of leprosy transmission. A previous study by Saeki *et al.* revealed that *M. leprae* existed on the surface of nasal cavities of residents in areas with high prevalence (6). Here, we report the distribution of different TTC genotypes of *M. leprae* among family members of each household and inconsistent genotypes obtained from patients and their family members in the same dwelling. The results strongly supported the previously proposed hypotheses (7) on the existence of an infectious source(s) other than that of patients living with family members.

Materials and Methods

Samples from patients

To clarify whether the TTC genotype in one patient varies or not, genotypes of the bacilli obtained from various lesions of one patient were compared. Slit-skin smear samples (SSS) from 45 lesions of 22 patients, two SSS from 21 patients and 3 SSS from one patient, were obtained at Central Special Skin Clinic (CSSC), Yangon General Hospital. Samples were collected in the same manner as is used for routine slit-skin smear testing for bacterial index examination. The sample on the disposable surgical blade was soaked in 70% ethanol and kept at room temperature until test.

Samples from patients and their contacts (who develop new case later) in the same dwelling

TTC genotypes of the bacilli from the lesion of four multi-family cases, multiple leprosy case among family member or living in the same dwellings, were compared. Skin slit samples were collected from at least two lesions of each patient. The genotype of each isolate was examined as described below.

Case 1: A MB case supposed to be a index case and his son developed the disease later.

Case 2: The same as Case 1 in another house.

Case 3: Supposed index MB case and after 10 months of MDT, his daughter developed the disease.

Case 4: MB case and after 9 months of MDT, his brother developed the disease

Samples from household contacts

TTC genotypes of the bacilli from nasal swab specimen of 92 household contacts (HC) in 18 dwellings with 22 patients diagnosed at CSSC were examined. HC were defined as persons sleeping during the night under the same roof. Nasal swabs were taken by introducing cotton tip swabs (sterilized *JCB* MENTIP, Japan) 2-3cm into each nostril successively, and rubbing gently on the lateral and median sides of each cavity. Swabs were immediately chilled (kept in ice box) and transported to the Immunology Research Division, DMR (Lower Myanmar) and analyzed.

Preparation of template DNA and sequencing analysis

The sample obtained from slit skin was removed

from the blade and collected as a pellet by centrifugation at 10,000 rpm for 20 min in 70% ethanol and then washed with phosphate-buffered saline. The template was prepared by treatment with lysis buffer according to the method described by Klatser *et al*, (8), and then the TTC genotype was examined.

Templates from nasal swab materials and slit-skin samples were prepared by treatment with lysis buffer at 60°C overnight as described elsewhere (8), TTC repeats regions were amplified by PCR with the primers indicated by Shin *et al*. (5). Copy numbers of TTC repeats were examined by the direct sequencing of the PCR products. Briefly, the regions flanking TTC repeats were amplified using a G mixture and a FailSafe PCR system (EPICENTRE, Madison, Wis. USA). DNA samples for sequencing were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden, Germany) after 1.2% Seakem agarosegel electrophoresis of PCR products. Samples were sequenced with a BigDye terminator cycle sequencing FS Ready Reaction kit Ver. 1.1 (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) and an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

Ethical approval

The study was approved by the Institutional Ethical Review Committee of Department of Medical Research (Lower Myanmar). Informed consent was obtained from all subjects. Bacillary samples of nasal swabs and slit-skin smears were collected after informed consent was obtained.

Results

Genotype of the bacilli from the nasal swab samples

Of 92 HC in 18 dwellings, there were 30 (33%) HC individuals carried the bacilli on the surface of their nasal cavities. TTC genotype of the bacilli from nasal mucus of HC in 14 dwellings out of 18 dwellings was identical. Genotype of the bacilli obtained in 4 multi-family cases different among family members (Table 1). Residents in these houses harbored different TTC genotypes from each other; their TTC genotypes were 9, 11, 12, 13, 14, 15, 16, 17, 21, and 22 repeats. The TTC repeats of the bacilli from the skin lesion of new MB case consisted of 11 copies and the TTC repeats of the bacilli from his nasal cavities was 15, on the other hand the bacilli from his family contacts, wife and son, showed 14 and 17 copies respectively. The TTC repeats genotype of the bacilli from PB patient showed 21 copies but bacilli from his household contact (HC) nasal mucus showed 15 copies. The TTC repeats of the bacilli from another new MB case consisted of 13 copies, but the bacilli from his family contacts, two daughters and a son, showed 13, 16 and 9 copies (Table.1). The frequency of each TTC genotype of the bacilli obtained from 45 skin lesion and 52 nasal samples from 22 patients and HC were shown in Table 2. The most predominant genotype was 16 copies of TTC repeats and the 2nd dominate type was 14 copies of TTC repeats.

Genotype of the bacilli in the lesions

From all 22 patients, 45 samples of different lesions showed identical genotypes. The most dominant genotype has 16 copies of TTC repeats in these patients. The other genotypes (number 9, 11, 12, 13, 14, 15, 16, 17, 21 and 22 copies of TTC repeats) were detected. The frequency of each TTC genotype observed in samples from lesions of the patients and the nasal cavities of the residents is shown in Table 2.

Comparison of TTC genotypes among patients in a dwelling

The TTC genotypes of *M. leprae* of supposed index and secondary cases were compared. The genotypes of index case patients in two multi-family cases harbored the bacilli with 13 and 22, and their son (secondary case) showed 9 and 17copies of TTC repeats respectively. In case 3 who was MB case harbored bacilli with 11 copies of TTC repeats, after 10 months of MDT his daughter developed as secondary case and harbored bacilli with 14 TTC repeats. Another case 4 of household cases of two brothers showed different TTC genotypes (15 and 16 TTC repeats) within the family (Table 3).

Discussion

Elucidation and understanding of the transmission mode, the source and the routes of transmission, of *M. leprae* are essential in developing drastic measures to prevent an infection. Previous sero-epidemiological studies indicated widespread *M. leprae* infections within a population (9, 10, 11, 12), and studies by PCR on the distribution of the bacilli also found that many individuals in areas in which leprosy is endemic carried *M. leprae* on the surface of their nasal cavities (6, 12, 13). These studies suggested the presence of an infectious source other than that of a patient within the same dwelling. The aim of this study was to clarify

Table 1. TTC genotypes of *M. leprae* detected from the skin and surfaces of nasal mucosa of patients and surfaces of nasal mucosa residents living in the same house.

Multi-family case	Leprosy patient (Type of patients)	Contacts (Relationship)	TTC genotype (Slit skin)	TTC genotype (Nasal swabs)
A	MB*		11	15
		Wife	-	14
		Son	-	17
B	PB**		21	18
		Grand mother	-	15
C	MB*		16	15
		Son	-	16
		Son	-	15
D	MB*		13	16
		Daughter	-	13
		Daughter	-	16
		Son	-	9

* MB; Multibacilary

**PB; Paucibacillary

microbiologically whether or not MB cases in the same dwelling represent the main source of infection. Establishing a methodology to discriminate the isolates of *M. leprae* is fundamental for these purposes. Although no useful genotyping methods for epidemiological analysis have been available until in 2000, two genomic divergence of *M. leprae* successfully found based on variable number tandem repeats (VNTRs) (5, 14). One of the authors (M. Matsuoka) discovered that *M. leprae* isolates could be divided into two subtypes on the basis of the polymorphism in the *rpoT* gene. The geographical distribution of each genotype in the world was biased and seemed to be related to prehistoric movement of the human race (14). Nevertheless, the genomic diversity of the *rpoT* cannot be used for epidemiological tracing of the transmission of leprosy bacilli. Genotyping by Single Nucleotide Polymorphisms (SNPs) is applicable to analyze movement of the human race but neither useful for analyzing transmission. (15). Genotyping to compare diversity of short-tandem-repeat loci on the basis of PCR is feasible for community based molecular epidemiological analysis, since *M. leprae* is not cultivable and shows very low levels of diversion in genomic DNA (16). Variety in the copy numbers of TTC repeats can be used to classify *M. leprae* into a considerable number of subtypes and discriminate isolates for each leprosy case. It is reasonable to assume that if the index case in the same dwelling is the source of infection, the genotypes detected in the house should be identical among the household members. In this study, various types of TTC genotypes were detected from nasal mucosa of the healthy HC.

Table 2. Frequency of each genotype observed in patients and household contacts.

No. of repeats	Genotype frequency		
	Patients lesion	Nasal mucus	Total
9	2	1	3 (3.1%)
11	2	1	3 (3.1%)
12	6	4	10 (10.3%)
13	6	6	12 (12.3%)
14	4	9	13 (13.4%)
15	4	8	12 (12.3%)
16	11	12	23 (23.7%)
17	2	4	6 (6.2%)
21	2	3	5 (5.2%)
22	6	4	10 (10.3%)
Total	45	52	97

Table 3. TTC genotypes of *M. leprae* obtained from multi-family cases.

Case	TTC genotype of the bacilli from supposed index case	TTC genotype of patient bacilli from patient secondary case
1	Father: 13 copies	Son:9 copies
2	Father:22 copies	Son:17copies
3	Mother: 11copies	Daughter:14 copies
4	Older brother:16copies	Younger brother: 15copies

Results obtained clearly demonstrated that there were families with different TTC genotypes of *M. leprae* on the surface of nasal cavities among the residents in the same dwelling. Therefore, the results of the investigation indicate that these residents are contaminated by bacilli with different genotypes. No variations in genotype among the isolates obtained from various lesions in the same patient were shown. This result consequently enables comparisons of the genotypes of bacilli obtained from different patients. We had identified the existence of TTC genotypes of *M. leprae* that differed between the newly detected family contacts and the supposed index case patient. These results strongly suggest that the bacilli did not originate from a single patient in the dwelling and also indicate the exposure of the family members

to infectious sources out of the dwelling. Previous sero-epidemiological studies suggested that for the majority of cases, the possible source of infection might be in the environment rather than in direct contact with leprosy patients (9, 14, 17). The findings by PCR, which revealed the wide distribution of the bacilli among the residents in endemic areas, also indicated that the transmission of the bacilli was not only from the leprosy patients (6, 12, 13, 17, 18). The present study strongly supports these assumptions respecting the infectious source(s). Although many epidemiological observations indicated that the household contact was the risk factor for the development of leprosy (18, 19), on the other hand, many new cases occurred among populate without any known contact with patients (20). Therefore the source of the secondary case is not only from his/her household. The tendency seen of the accumulation of patients in some families might be attributed to other conditions such as susceptibility to leprosy infection, which is related to genetic predisposition as well as to acquired factors (21). Two groups of the household leprosy cases showed apparently different TTC genotypes between a father and his son, mother and daughter and among brothers. The inconsistency of the genotypes between *M. leprae* isolates obtained from household cases of patients living in the same dwelling clearly indicates that these patients are not always the source in infections of the other family members.

Though the members of the other groups of leprosy cases other than 4 cases shown in table 3 showed the same genotype, whether those people were truly infected by the patient in the house was unclear. The presence of the same genotype in two cases doesn't necessarily imply the infection was occurred from a patient to family contacts, for some TTC genotypes such as 16 copies were widely distributed in the areas. Other polymorphisms which can discriminate within a given TTC genotype are needed to elucidate this problem. Better epidemiological analysis could be done by the combination of various genotyping techniques. However, TTC genotyping enabled the subtyping of *M. leprae* into more types than *rpoT* or SNPs genotyping. Other short polymorphic-tandem-repeat loci exist in *M. leprae* genome (2, 22) combination with genotyping using other polymorphisms might be a useful tool for precise epidemiological analysis especially for the strains with same TTC copy numbers. Other genotyping measures depending on other short polymorphic tandem repeat loci are proposed (3).

The frequency of 24 or 25 TTC repeats was the highest in the previous study, which examined *M. leprae* isolates obtained in Cebu, Philippines (5). Bacilli with 10 copies of TTC repeats were most frequently isolated in the present study, and the bacilli with large numbers (such as 37) of TTC repeats were not detected (Table 3). It is of interest to compare the frequencies of each genotype in different areas, since the results of a previous study indicated that the spread of the bacilli with specific genotypes was consistent with migration of some human groups (14, 23). The evidence resulting from the present molecular epidemiological study indicated the existence of an infectious source other than patients in the same dwelling. Wide distribution of the bacilli among residents (6, 8, 12) and a high positive ratio of anti-PGL-1 antibody among healthy residents (10, 11) suggested that the bacilli existed in certain sources to which people were commonly exposed. Genotyping study of the bacilli obtained at the areas with high leprosy prevalence also suggested infection other than patients (17, 18) Taking these results into consideration, the environment seems to be the most likely infectious source as suggested previously (24). However, it has not been elucidated so far.

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未感作T細胞を強く活性化するウレアーゼ欠損HSP70-MMP-II融合蛋白産生性リコンビナントBCGの作出

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はじめに

らい菌に対する生体防御反応は、インターフェロンガンマー（interferon gamma, IFN-γ）産生性タイプ1 CD4陽性T細胞とタイプ1 CD8陽性T細胞によって営まれている。これらのT細胞の活性化は、らい菌あるいはらい菌由来抗原を取り込んだ樹状細胞（dendritic cell, DC）との相互作用によって誘導される。樹状細胞が効率的にT細胞を活性化するためには、らい菌由来の主要抗原が効率的に用いられる必要があるが、らい菌の主要抗原の一つとして我々はmajor membrane protein (MMP)-II (Gene name, *bfrA* or ML2058)を同定し報告してきた。MMP-IIは、toll like receptor (TLR2)に結合する能力を有しNF-κBを活性化する[1]。そのため、MMP-II蛋白をパルスした樹状細胞は抗原特異的に未感作及びメモリータイプCD4陽性T細胞及びCD8陽性T細胞を活性化する[1]。さらに、MMP-IIは少菌型ハンセン病患者では末梢T細胞によって認識され得る蛋白であることも判明している[2]。一方で、WHOが推奨するMDT療法によって登録ハンセン病患者数は激減したが、新規ハンセン病患者数は著明な減少は示していない。したがって、ハンセン病の制圧には有効に作用するワクチンは不可欠である。歴史的には、地域によっては*Mycobacterium bovis* BCG (BCG)がワクチンとして用いられた時代があったが、有効性は最終的に26％であると結論されている[3]。BCGが有効に作用しない最大の理由は、BCGには固有の欠点、すなわち抗原提示細胞（antigen presenting cell, APC）に感染するとファゴゾームを形成し、ライソゾームとの融合を阻止することに起因している[4-6]。一方、BCGはらい菌などの病原性抗酸菌に共通して存在する抗原を有し、タイプ1 CD4陽性T細胞を活性化する能力を弱いながらも有していることが知られている。したがって、BCGに代わる新しい信頼性に富んだワクチンを作製するためには、BCGに改良を加えるのが一方法であると考えられる。著者等は、これまでにBCGの改良にあたり様々な努力を払ってきた。最初に、MMP-IIを細胞内で分泌するリコンビナントBCG（BCG-SM）を作製した[7]。BCG-SMは未感作のCD4陽性・CD8陽性T細胞をある程度活性化し、マウス足蹠に感染させたらい菌の増殖を不完全ながら抑制した[8]。こうした試みは、抗原提示細胞で主要抗原を分泌させる方策は抗原提示細胞及びT細胞を活性化する上で有効であることを証明していると考えられる。そこで、MMP-IIを細胞内で分泌させるにあたり、シャペロン効果を有するheat shock protein (HSP)70を利用する、すなわちHSP70-MMP-II融合蛋白を分

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泌させることにして、第２のリコンビナントBCG（BCG-70M）を作製した[9]。BCG-70Mは、BCG-SMに比しより強くヒト未感作CD4陽性T細胞及びCD8陽性T細胞を活性化し、IFN-γの産生を誘導した[10]。さらに、第３のリコンビナントBCG（BCG-ΔUT-11-3）を作製した[6]。BCGはウレアーゼを保有するため尿素からアンモニアを産生し、ファゴゾームのpHをアルカリ側に傾けることで、ファゴゾームの酸性化を抑制し、ライソゾームとの融合を阻止している[4-5]。そこで、BCGからウレアーゼをコードする*UreC*遺伝子を除去しBCG-ΔUT-11-3を作製した。BCG-ΔUT-11-3は容易にライソゾームへ移行し、ヒト未感作CD4陽性T細胞を強く活性化した[6]。したがって、二つの独立した方策、HSP70-MMP-II融合蛋白の細胞内分泌とウレアーゼ活性の除去は、ともにT細胞を活性化する上で有効であった。そこで、この二つの方策を組み合わせる、すなわちウレアーゼ欠損リコンビナントBCGにHSP70-MMP-II融合遺伝子を導入して第４のリコンビナントBCG（BCG-D70M）を作製した（図１）。BCG-D70MのT細胞活性化能を評価するにあたり、コントロールBCGとしてBCG-261H（ベクターコントロールBCG）、BCG-70M及びBCG-ΔUT-11-3を用いた。

BCG-D70Mの作出

BCG-ΔUT-11-3は、BCG-Tokyo株を親株として用い作製した。ウレアーゼは、BCGゲノム上の*ureABCFGD*の６個の遺伝子から作られる。その中で、最も長い*ureC*遺伝子（1734bp）を破壊する遺伝子の標的とした。また、外来遺伝子をBCG菌体内へ導入する方法として、抗酸菌に感染するウイルスであるファージの中で温度感受性変異株を利用する方法を用いた。本法は、これまで知られていた方法より効率的に外来遺伝子を導入し得る方法である。ハイグロマイシン耐性遺伝子を挟むように*ureC*遺伝子の上流および下流の塩基配列をプラスミドpYUB854へクローニングした。さらに迅速発育抗酸菌*M.smegmatis*を用い*ureC*破壊用ファージの調整を行った。組換えファージをBCG-Tokyoに感染させ、ハイグロマイシンを含む平面培地に播き30℃で培養を行った。約３週間後、形成されたコロニーを培養し、ウレアーゼ試験が陰性であることを確認した。ゲノムに組込まれたハイグロマイシン耐性遺伝子を切り出し、親株と同じカナマイシン、ハイグロマイシン感受性の株ΔUT-11-3を選択した。BCG-D70Mは、ΔUT-11-3

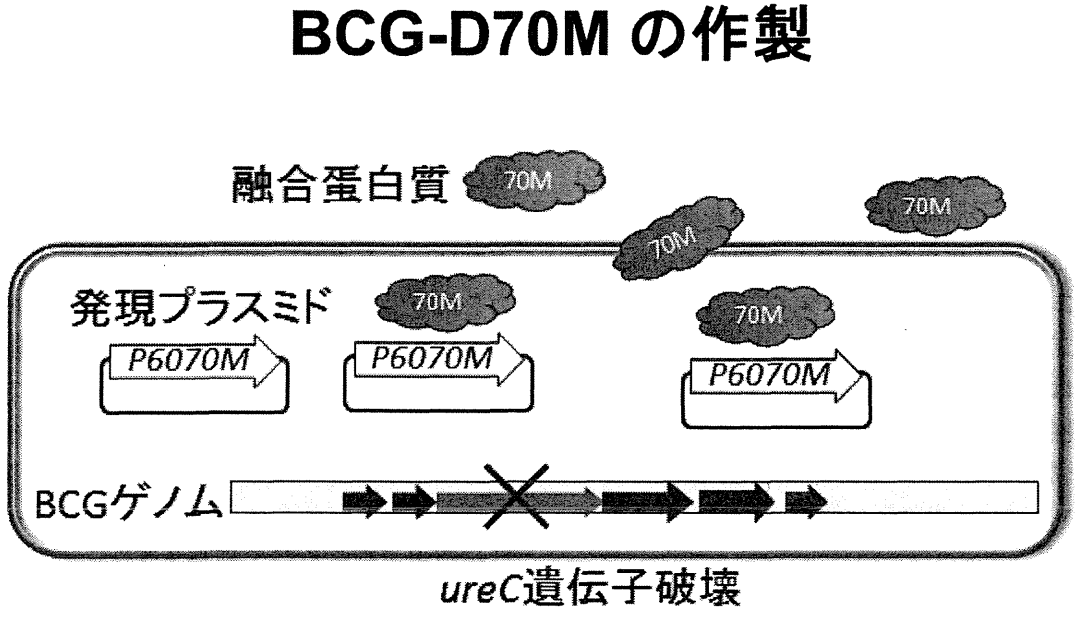

図１：ウレアーゼをコードするUreC遺伝子を欠損するリコンビナントBCG（BCG-ΔUT-11-3）に、HSP60プロモーター制御のもとBCG由来HSP70遺伝子とらい菌由来MMP-II遺伝子を連結した融合遺伝子を導入し、新規リコンビナントBCG（BCG-D70M）を作製した。

へ*hsp60*のプロモーター領域の下流にBCG*hsp70*遺伝子とらい菌*MMP-II*遺伝子を融合させ、抗酸菌体内でプラスミドとして維持するpMV261に組み込み、さらに、菌体内での安定した維持のためハイグロマイシン耐性遺伝子を持つプラスミドを導入して作製した。BCG-D70Mは、菌体培養液中にHsp70とMMP-IIの融合した形（HSP70-MMP-II融合蛋白）を分泌することをウエスタンブロット法を用い確認した。

BCG-D70Mの評価

BCG-D70Mは菌体外へHSP70-MMP-IIを分泌し、コントロールBCGに比し最も強く樹状細胞を刺激してIL-12p70を産生誘導するとともに、IL-1β及びTNFαを効率的に産生誘導した。さらに、樹状細胞に感染させるとBCG-261Hに比し有意にHLA-ABC・HLA-R・CD86及びCD83抗原の発現を増強させた。また、BCG-D70Mを感染させた樹状細胞を抗原提示細胞として用いると、ヒト未感作CD4陽性T細胞及びCD8陽性T細胞を抗原特異的に活性化してIFN-γの産生を誘導した。BCG-70M・BCG-ΔUT-11-3は、マクロファージを介してヒトのメモリーCD4陽性T細胞を活性化し得なかったが、BCG-D70Mはメモリー CD4陽性T細胞を活性化してIFN-γの産生を誘導した。この活性化も抗原特異的であった。BCG-D70MによるT細胞活性化機構を解明するため、BCG-D70M感染樹状細胞の細胞表面を解析すると、MMP-II由来のペプチドが発現していた。したがって、BCG-D70MがファゴゾームでHSP70-MMP-II融合蛋白を分泌し、分泌された融合蛋白が効率的にプロセッシングされていると考えられた。さらに、樹状細胞を予めファゴゾームの酸性化抑制剤であるクロロキニンで処理しておくと、樹状細胞表面のMMP-II由来分子の発現が消失することから、ファゴゾームの成熟化が樹状細胞表面へのMMP-IIの発現に関与していることが判明した。そこで、ファゴゾームの成熟化とT細胞の活性化との関連を検討するため、樹状細胞及びマクロファージを予めクロロキニンで処理した後BCG-D70Mを感染させT細胞を刺激すると、未感作CD4陽性T細胞・未感作CD8陽性T細胞及びメモリーCD4陽性T細胞からのIFN-γの産生が全て有意に抑制されることが判明した。さらに、樹状細胞をブレフェルディンAあるいはラクトシスティンで処理すると未感作CD8陽性T細胞の活性化が抑制された。つまり、BCG-D70MはTAP及びプロテオゾームに依存したcytosolic cross-presentation pathwayによって未感作CD8陽性T細胞を活性化していることが明らかとなった。また、BCG-D70Mは*in vitro*で$CCR7^{low}$及び$CD27^{low}$のメモリータイプCD8陽性T細胞及びパーフォリン産生性キラーCD8陽性T細胞を効率的に産生し、かつC57BL/6マウスにBCG-D70Mを皮下接種すると、*in vitro*でMMP-IIあるいはHSP70といったリコール抗原に反応してIFN-γを産生するメモリーT細胞を効率的に産生した。このメモリーT細胞産生能は、皮下接種後12週間以上継続した。最後に、BCG-D70Mのワクチン効果をC57BL/6マウスを用いて検証すると、ベクターコントロールBCGに比し効率的かつ有意に足蹠へ接種されたらい菌の増殖を抑制し、充分なるワクチン効果を有していることが判明した。

おわりに

ハンセン病に対し有効に作用する改良型BCGの作出にあたっては、ウレアーゼ活性の除去、HSP70-MMP-II融合遺伝子の導入が有効であった。信頼されるハンセン病ワクチンの確立には更なる検討と改良技術の導入が必要であるが、今回研究で得られた知見が一助となることを願っている。

ハンセン病学会賞の受賞にあたっては、共著者及びハンセン病のワクチン開発に携わり多くの助言と協力を頂いたハンセン病研究センター内外の諸先生に厚く御礼申し上げたい。

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Enhanced activation of T lymphocytes by urease-deficient recombinant bacillus Calmette-Guérin producing heat shock protein 70-major membrane protein-II fusion protein

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Key words : leprosy, MMP-II, recombinant BCG, T cell, vaccine

To activate naïve T cells convincingly using *Mycobacterium bovis* BCG (BCG), rBCG (BCG-D70M) that was deficient in urease, expressed with gene encoding the fusion of BCG-derived heat shock protein (HSP) 70 and *Mycobacterium leprae*-derived major membrane protein (MMP)-II, one of the immunodominant Ags of *M. leprae*, was newly constructed. BCG-D70M was more potent in activation of both $CD4^+$ and $CD8^+$ subsets of naïve T cells than rBCGs including urease-deficient BCG and BCG-70M secreting HSP70-MMP-II fusion protein. BCG-D70M efficiently activated dendritic cells (DC) to induce cytokine production and phenotypic changes, and activated $CD4^+$ T cells even when macrophages were used as APCs. The activation of both subsets of T cells was MHC and CD86 dependent. Pre-treatment of DC with chloroquine inhibited both surface expression of MMP-II on DC and the activation of T cells by BCG-D70M-infected APCs. The naïve $CD8^+$ T cell activation was inhibited by treatment of DC with brefeldin A and lactacystin so that the T cells was activated by TAP- and proteosome-dependent cytosolic cross-priming pathway. From naïve $CD8^+$ T cells, effector T cells producing perforin and memory T cells having migration markers, were produced by BCG-D70M stimulation. BCG-D70M primary infection in C57BL/6 mice produced T cells responsive to *in vitro* secondary stimulation with MMP-II and HSP70, and more efficiently inhibited the multiplication of subsequently challenged *M. leprae* than vector control BCG. These results indicate that the triple combination of HSP70, MMP-II and urease depletion may provide useful tool for inducing better activation of naïve T cells.

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Impact of Amino Acid Substitutions in B Subunit of DNA Gyrase in *Mycobacterium leprae* on Fluoroquinolone Resistance

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Abstract

Background: Ofloxacin is a fluoroquinolone (FQ) used for the treatment of leprosy. FQs are known to interact with both A and B subunits of DNA gyrase and inhibit supercoiling activity of this enzyme. Mutations conferring FQ resistance have been reported to be found only in the gene encoding A subunit of this enzyme (*gyrA*) of *M. leprae*, although there are many reports on the FQ resistance-associated mutation in *gyrB* in other bacteria, including *M. tuberculosis*, a bacterial species in the same genus as *M. leprae*.

Methodology/Principal Findings: To reveal the possible contribution of mutations in *gyrB* to FQ resistance in *M. leprae*, we examined the inhibitory activity of FQs against recombinant DNA gyrases with amino acid substitutions at position 464, 502 and 504, equivalent to position 461, 499 and 501 in *M. tuberculosis*, which are reported to contribute to reduced sensitivity to FQ. The FQ-inhibited supercoiling assay and FQ-induced cleavage assay demonstrated the important roles of these amino acid substitutions in reduced sensitivity to FQ with marked influence by amino acid substitution, especially at position 502. Additionally, effectiveness of sitafloxacin, a FQ, to mutant DNA gyrases was revealed by low inhibitory concentration of this FQ.

Significance: Data obtained in this study suggested the possible emergence of FQ-resistant *M. leprae* with mutations in *gyrB* and the necessity of analyzing both *gyrA* and *gyrB* for an FQ susceptibility test. In addition, potential use of sitafloxacin for the treatment of problematic cases of leprosy by FQ resistant *M. leprae* was suggested.

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Introduction

Leprosy is one of the oldest human infectious diseases and remains a public health problem. At the beginning of 2011, the number of registered leprosy cases was 192,246, and that of new cases reported during 2010 was 228,474, mainly from Asian, Latin American, and African countries [1]. Multibacillary leprosy is usually treated by administering dapsone (DDS), clofazimine (CLF), and rifampicin (RIF) in combination, where single skin lesion paucibacillary leprosy is recommened to be treated by administering RIF, ofloxacin (OFX), and minocycline (MIN) [2]. Since the late 1990s, multi-drug resistant (MDR) isolates of *M. leprae*, resistant to RIF and DDS, have emerged and the importance of OFX has been a focus for the treatment of MDR-leprosy [3]; however, their use not only for leprosy but also for other infectious diseases including tuberculosis has already led to OFX resistance in *M. leprae* [4–8]. Hence, early prediction of FQ resistance seems to be essential for the proper treatment of leprosy.

OFX is a fluoroquinole (FQ) and FQs inhibit type II DNA topoisomerases, including DNA gyrase and topoisomerase IV [9]. FQ resistance is given mainly by amino acid substitutions in the quinolone resistance-determining regions (QRDRs) located on the N- and C-terminal domains of A (GyrA) and B (GyrB) subunits of DNA gyrase and, less prominently, amino acid substitution in the QRDR on the N- and C-terminal domains of A (ParC) and B (ParE) subunits of topoisomerase IV has been reported [10]. *M. leprae* has only DNA gyrase [11], which is therefore the sole target of FQs. Genetic analysis of *M. leprae* clinical isolates revealed reduced FQ sensitivity associated with amino acid substitutions only at position 89 or 91 and 205 in GyrA and GyrB, respectively [4–8,12]. In the latter study, the contribution of amino acid

Author Summary

Leprosy is one of the oldest human infectious diseases, which remains a public health problem with more than 200,000 new cases every year worldwide. Since the late 1990s, multi-drug resistant leprosy, resistant to rifampicin and dapsone, has emerged and the importance of ofloxacin has increased. However, their use for leprosy and other infectious diseases has already elicited ofloxacin resistant leprosy cases. Hence, early detection of ofloxacin resistance is essential for proper treatment. This study, by utilizing recombinant technology, predicted the future emergence of ofloxacin resistant *Mycobacterium leprae* with mutations that have not yet been reported. The data are useful for predicting ofloxacin resistance and, hence, able to contribute to the proper treatment of leprosy through suggesting the importance of analyzing gene mutations for FQ susceptibility testing.

substitution in GyrA at position 89 or 91 to reduced FQ sensitivity was confirmed by an *in vitro* analysis [13]. In addition, the effect of amino acid substitution at position 95 in GyrA was predicted [14]. In contrast, amino acid substitution in GyrB at position 205, reported by You et al. [8], was revealed not to affect FQ sensitivity by an *in vitro* study [13]. Reduced FQ sensitivity associated with amino acid substitutions has been frequently reported in GyrA in *M. tuberculosis*; however, those in GyrB have been reported less frequently (Figure 1) [10,15]. According to the reports, important residues of GyrB in *M. tuberculosis* were thought to be at codon 461, 499 and 501 (with a counting system proposed by Maruri et al. [10]). Notably, amino acid substitutions at position 499 and 501 in *M. tuberculosis* showed a correlation with reduced FQ susceptibility by an *in vitro* assay [15 18]. Lack of the detection of FQ-resistant *M. leprae* carrying GyrB amino acid substitutions is due to the low number of FQ resistant cases analyzed. Hence, it is highly important to elucidate the contribution of amino acid substitutions

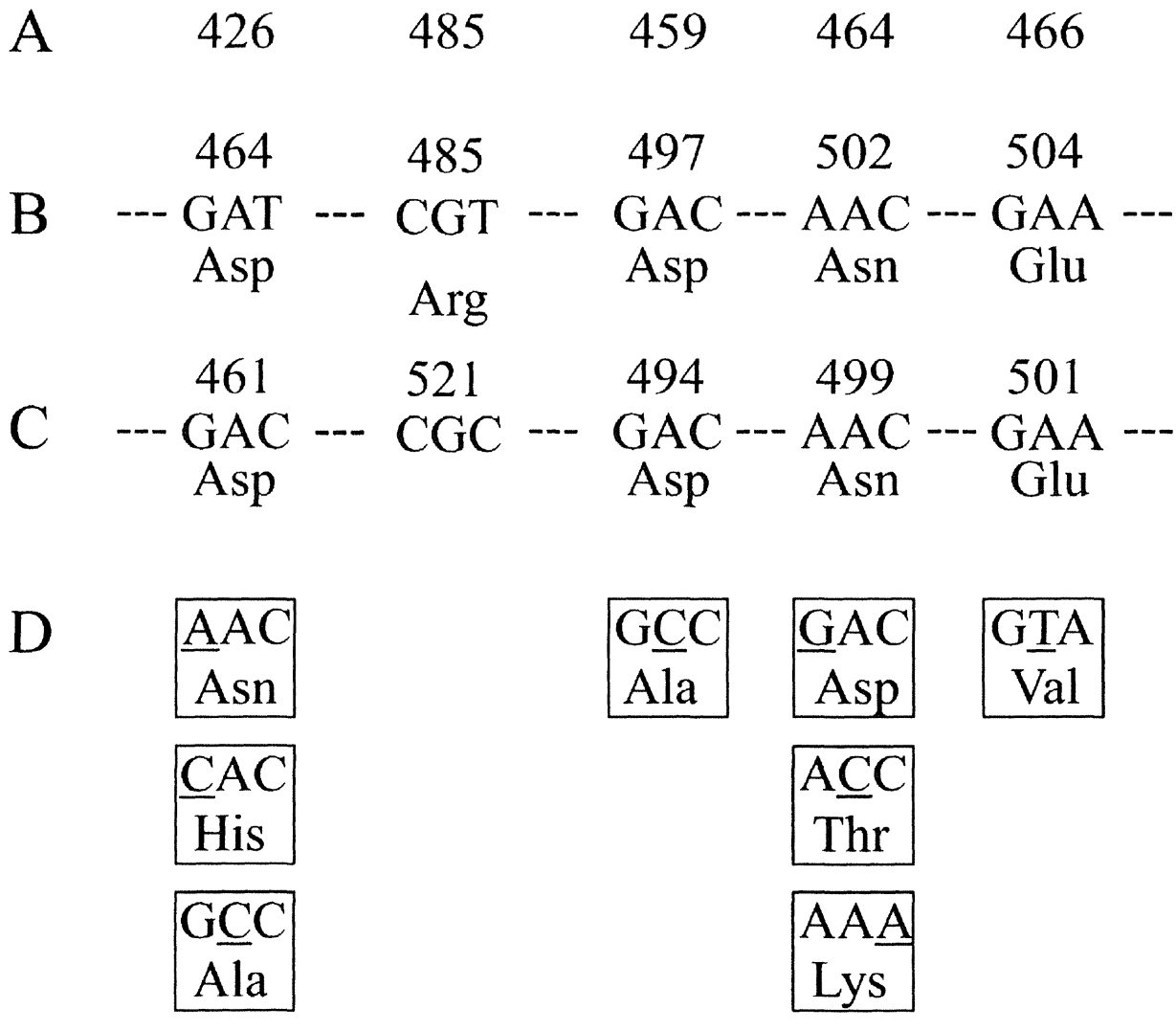

Figure 1. Nucleotide and amino acid sequences of QRDR of *M. leprae* and *M. tuberculosis gyrB* and mutations found in FQ-resistant isolates. (A) amino acid number of GyrB in *E. coli*, (B) Amino acid number, nucleotide sequences and amino acid sequence of WT *M. leprae* GyrB QRDR, (C) Amino acid number, nucleotide sequences and of WT *M. tuberculosis* GyrB QRDR, (D) Altered amino acids and corresponding nucleotide substitutions found in higher rate in FQ-resistant *M. tuberculosis* isolates.
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